Biomarker considerations in monitoring petrogenic pollution using the mussel *Mytilus galloprovincialis*

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Abstract

Mussels are worldwide bioindicators in pollution monitoring since they fulfil the requirements for being good sentinels. However, some methodological concerns arise in the use of particular biomarkers, particularly those displaying low enzymatic rates and/or limited responsiveness to chemicals and biological-related variability. In the present study, the suitability of oxidative stress and detoxification parameters when using mussels as sentinels of polycyclic aromatic hydrocarbons (PAHs) pollution is addressed. Present results show that the S9 subcellular fraction of the digestive gland in mussels is an adequate and convenient matrix where to measure most pollution related biomarkers. Furthermore, this work constitutes the first evidence of the potential suitability of using particular carboxylesterase (CE) activities in determining PAHs exposure in mussels. This fact could imply the replacement of more controversial cytochrome P450 components (phase I oxidation), which are only measurable in microsomal fractions, by CEs (measured in S9 fractions) as good alternatives for phase I reactions in PAH-exposed mussels. Some methodological considerations, such as the need of including commercial purified proteins in biomarker determinations for quality assurance, are evaluated.

Keywords: Mussels, antioxidant enzymes, CYPs, carboxylesterases, PAHs, biomarkers

Introduction

Marine ecosystems are the final sink for many land-based chemicals but also from activities carried out in their waters such as transportation, spillages and aquaculture. This raises concerns regarding the toxicological consequences of those compounds on aquatic wildlife and cultured species (Nilsen et al., 2019; Tornero and Hanke, 2016). Legacy persistent organic pollutants (POPs) and polycyclic aromatic hydrocarbons (PAHs) are among the chemicals that are most commonly quantified to trace anthropogenic pollution (Viñas et al., 2012; Viñas et al., 2018). Nonetheless, there is a broad range of new drugs, known as emerging contaminants or contaminants of environmental concern, which have been more recently introduced into the wastewater systems and are likely to cause long term chronic effects (Fabbri and Franzellitti, 2016; Mezzelani et al., 2018). Due their inefficient depuration or their recalcitrant properties, some of these products can also reach marine systems (Sanchez-Avila et al., 2009).

Mussels are long-recognised sentinels in marine pollution monitoring programs given that they have properties that enable them to integrate and reflect local chemical pollution while allowing measuring the corresponding biochemical and physiological responses (Cajaraville et al., 2000; Martinez-Gomez et al., 2017). Mussel digestive glands (Dallares et al., 2018; Gonzalez-Rey and Bebianno, 2014; López-Galindo et al., 2014) or whole organisms (Freitas et al., 2017, 2019), have been used to study the effects of pollutant exposures in both laboratory and field conditions. However, methodologically speaking, there is the need to validate the biochemical parameters measured for inter-calibration exercises as well as to validate the quality of the data obtained from large scale measurements. This is especially relevant since some enzymatic activities in bivalves, and mussels in particular, are lower than in other groups of bioindicator species such as fish (Livingstone, 1998; Hanson et al., 2017). For

example, even if antioxidant defences and phase II conjugation enzymes such as glutathione *S*- transferases (GSTs) are well established biomarkers in mussels (Regoli and Giuliani, 2014), this is not the case for cytochrome P450 related activities (CYPs). Fluorometric-based CYP assays are commonly used in fish studies, and despite the existence of CYP-like related activities in freshwater (Aguirre-Martinez et al., 2015; Faria et al., 2009) and marine bivalves other than *Mytilus galloprovincialis* (Falfushynska et al., 2018; Maranho et al., 2015; Pereira et al., 2012), their activities in molluscs are relatively low and sometimes doubted to be inducible by Aryl hydrocarbon receptor (AhR) agonists (Butler et al., 2001). For this reason, some authors question the use of measuring CYP-like catalytic activity in invertebrates (Hahn, 2002) and in the marine mussel *M. galloprovincialis* in particular (Faria et al., 2009). Other (well represented) metabolising enzymes in bivalves might be good alternatives to explore. We hypothesize that this is the case of phase I carboxylesterases (CEs), involved in the hydrolysis of endogenous compounds but also in the detoxification of many exogenous chemicals including pesticides and drugs in mammalian systems (Satoh and Hosokawa, 1998; Satoh and Hosokawa, 2006; Wheelock et al., 2008; Fukami et al., 2010; Fukami and Yokoi, 2012; Imai et al., 2006). Recent studies with bivalves have demonstrated their *in vivo* ability to metabolise drugs such as the retroviral Tamiflu® (Dallares et al, 2019) and their *in vivo* response to environmental pesticides (Dallares et al., 2018) as well as *in vitro* sensitivity to pesticides and drugs including plastic additives (Sole et al., 2018a; Sole et al., 2018b; Sole and Sanchez-Hernandez, 2018; Nos et al., 2020).

In the context of the increasing need to determine the degree of anthropogenic impact of marine systems, for which PAHs quantification is commonly used, the present study aimed: i) to facilitate monitoring programs by identifying the cellular fraction on which most biomarkers could be adequately measured; ii) to determine the

suitability of using phase I (e.g. CEs) as biomarkers in mussels chronically exposed to PAHs as alternatives to CYP-related catalytic activities, whose relevance in bivalves is frequently discussed and iii) to propose some methodological improvements when using traditional biomarkers in pollution monitoring with mussels as sentinels (i.e. the inclusion of purified proteins in the measures to validate protocols and allow large scale study comparisons).

Material and methods

Mussel collection

Mussels, *Mytilus galloprovincialis*, aimed for chemical and biochemical determinations were collected from the relatively PAH-free region of the Ebre Delta (NE Iberian Peninsula; Mediterranean) at coordinates 40.622383; 0.668552 from aquaculture farms devoted to their commercialization. For the same purpose, wild specimens were additionally collected from the Barcelona harbour area (coordinates 41.377508; 2.185741), where natural populations are found despite the area being chronically polluted by PAHs. Additional samples were collected from this site to search for size-activity relationships (n=22). Samplings took place at the same time period (October-November 2017). Mussels from both locations were transported to the laboratory under cold conditions (~4ºC using ice blocks) and immediately dissected before their use in the experimental procedures described below.

Sample processing

Selection of the most suitable subcellular fraction

With the aim selecting a single subcellular fraction on which most of the potential pollution biomarkers could be analysed, a total of 24 animals were collected at the Ebre Delta (PAH-free reference site) and six pools were made using 4 digestive glands in each. Pools were used in this specific case to ensure having enough microsomes to conduct the analyses. The S9 fraction was obtained by homogenizing the samples in a phosphate buffer (100 mM, pH 7.4) containing 150 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1mM dithiothreitol (DTT) at a 1:5 (w:v) ratio and centrifuging at 10,000 x g for 30 min. Resulting homogenates were further centrifuged at 100,000 x g for 1 h to yield the cytosolic (supernatant) and microsomal (pellet) fractions as described in more detail by Sole and Livingstone (2005). Analyses were carried out (when possible) in each of the three fractions (cytosol, S9 and microsomes) in search of the most appropriate fraction for each assay. Antioxidant and CE activities were best measured in the S9 fraction (where results showed the lowest deviations and coefficient of variation) and thus this fraction was selected for the site comparison study (see section 2.2.2). Cytochrome P450 associated reductase activities were only measurable in the microsomal fraction while CYP-related catalytic measures were only attempted in the microsomal fraction. Thus, these microsomal parameters were not further considered in the site comparison study.

PAH-related site contrasts

Individual digestive glands from mussels obtained at both (PAH-polluted and pristine reference) sites were dissected and immediately flash frozen individually in liquid nitrogen and stored at -80ºC until analysis (N=8 per site). Attention was paid to select animals of similar sizes to perform contrasts in bioaccumulation and enzymatic

activities. For each sample, the S9 fraction was obtained following the protocol detailed before.

Biochemical determinations

Enzyme activity determinations were carried out in mussels of similar size (4.6 ± 0.21 and 4.7 ± 3.0 cm) to avoid the influence of biological traits in biomarker determinations. Antioxidant activities and CE measures were carried out spectrophotometrically on S9 fractions of digestive glands. For the first, measurements consisted on well adopted protocols for determinations for catalase (CAT) (Aebi et al., 1974), glutathione reductase (GR) (Carlberg and Mannervik, 1985), glutathione peroxidase (GPX) (Günzler and Flohe, 1985) and glutathione *S*-transferases (GSTs) (Habig et al., 1974). CEs were measured using 4 different commercial substrates: ρ-nitrophenyl acetate (ρNPA), ρ-nitrophenyl butyrate (ρNPB), α-naphthyl acetate (αNA) and α-naphthyl butyrate (αNB). The formation of ρ-nitrophenol by ρNPA and ρNPB was recorded at 405 nm as described in Hosokawa and Satoh (2005) and naphthol by αNA and αNB was measured at 235 nm according to the Mastropaolo and Yourno (1981) method. All protocols are described in more detail elsewhere (Dallares et al., 2018). Activities were all expressed nmol · min^{-1} · mg protein $^{-1}$ except for CAT were results were expressed as μmol · min^{-1} · mg protein $^{-1}$. Protein content was determined for each sample as described further below.

To ensure linearity of former measures following the adopted protocols, an 8-point concentration range of commercial purified proteins was considered for each enzymatic assay carried out which is the same methodological procedure as for the bivalve S9 biomarker determinations (see Table 1).

Microsomal CYP-related determinations consisted in the following essays: i) reductase activities, measured spectrophotometrically at 550 nm using NAD(P)H cytochrome c reductase and NADH ferrycyanide reductase as described by Sole and Livingstone (2005); ii) catalytic O-deethylase activities of digestive gland CYPs, determined using fluorescent CYP-mediated substrates (ER: 7-ethoxyresorufin, PR: 7-pentoxyresorufin, BR: 7-benzyloxyresorufin, MR: methoxyresorufin; CEC: 3-cyano-7-ethoxycoumarin); iii) O-debenzyloxylase activity (BFCOD), using BFC (7-benzyloxy-4-trifluoromethylcoumarin) and iv) O-debenzylase activity using DBF (dibenzylfluorescein). All assay conditions were adapted from fish studies and used 50 µL of mussel microsomes. To ensure that measurements could be attributable to CYP activity, an inhibition study was carried out using the broad CYP inhibitor ketoconazole. For this, microsomes were incubated for 30 min at room temperature with one of three concentrations of ketoconazole (0.1, 1 and 10 µM) as described in detail by Koenig et al. (2013).

Total protein content of the different subcellular samples was determined by the Bradford method (Bradford, 1976) adapted to microplate, using the Bradford Bio-Rad Protein Assay reagent and bovine serum albumin (BSA; 0.05-1 mg/mL) as standard. Absorbance was read at 595 nm.

All assays were carried out in a TECAN Infinite 200 microplate reader in 96-well plates in triplicate at 25 ºC except for CYPs assays which were run at 30 ºC. Only linear reactions were considered and these were registered using the kinetic assays mode of Magellan V6.0 data analysis software.

Chemical analysis

A pool of about 10 g of whole soft tissue (corresponding to 5-6 mussels) was used for chemical characterisation and quantification of PAHs. The following PAHs were quantified in mussels from both pristine and polluted sites: Phenanthrene (Phe), Anthracene (Ant), Fluoranthene (Flu), Pyrene (Pyr), Benzo(a)anthracene (BaA), Chrysene (Chr), Benzo(e)pyrene (BeP), Benzo(b)fluoranthene, Benzo(b)fluoranthene (BkF), Benzo(a)pyrene (BaP), Benzo(ghi)perylene (BghiP), dibenz(ah)anthracene dB(ah)A and Indeno(1,2,3-c,d)pyrene (IP). These correspond to 12 out of the 16 PAHs that the Environmental Protection Agency (US-EPA) recommends to monitor (Keith 2014). The methodology used for their quantification consisted of Soxhlet extraction following HPLC with fluorescence detection and with the use of reference materials for quality assurance as described in detail elsewhere (Viñas et al., 2018). The limit of quantification was 0.25 ng/g wet weight (w.w.).

Statistics

Two pair comparisons were made using Student's t-test contrast after confirmation of parametric requirements (Shapiro-Wilk and Levene's tests for normality and homocedasticity of datasets, respectively). Correlation between biomarkers was made using Pearson's correlation coefficient. Statistical analyses were carried out using SPSS System Software v24 and the significance level for data analyses was set at $\alpha=0.05$. Data are presented as means ± SEM (standard error of mean).

Results and discussion

Mussel chemistry and enzymatic activities

206 The Ebre Delta is considered a pristine region as far as PAHs is concerned,
207 while the Barcelona harbour is an area historically described as being heavily loaded
208 with petrogenic PAHs (Porte et al., 2001). This was here confirmed through the
209 chemical analysis of collected mussels, showing that the Σ13 PAHs reached 9 ng/g at
210 the Ebre Delta and up to 565 ng/g at the Barcelona harbour (values in w.w.). The later
211 value is over 5-fold higher than the 100 ng/g w.w. typically considered to correspond to
212 heavily industrialised (and hence heavily polluted) areas (Viñas et al., 2009). Also, the
213 calculation of diagnostic ratios between PAHs allowed us identifying their origin:
214 petrogenic PAHs would include crude oils and refined crude oils such as gasolines,
215 heating oils, coals or asphalt, while pyrogenic substances are the result of fires, internal
216 combustion engines or furnaces. For instance, a Flu/(Flu+Pyr) ratio lower than 0.4
217 indicates petrogenic origin and it was much lower in the harbour mussel samples (0.05)
218 than in the Ebre region mussels (0.4). Another ratio, Ant/(Ant+Pyr), sheds light on this
219 subject and while ratio values scoring below 0.1 is suggestive of a petrogenic origin,
220 values over 1 indicate a pyrolytic source of PAHs. In the Barcelona harbour mussels
221 this value was 0.09. Altogether, data are supportive of the PAHs of the harbour being of
222 the petrogenic source. Detailed chemical results are presented as supplementary
223 material S1.

224 Since seasonality and animal size are factors described to affect biomarker
225 activities, including some of the analysed here (Banni et al., 2009; Cravo et al., 2013;
226 Uluturhan et al., 2019), only individuals from similar size and collected at the same time
227 period were considered in the study. The enzyme activities of mussels S9 digestive
228 gland fraction from these two sites showed differences in terms of antioxidant activities
229 (Table 2). The GR, t-GPX and GSTs (antioxidant) activities were significantly increased
230 in mussels from the harbour sampling site ($p<0.05$). Antioxidant enzymes are frequently

related to PAH exposure in bivalves (Regoli and Giuliani, 2014). CE-related activities (Table 2) showed substrate dependent responsiveness: while nitrophenyl substrates (ρNPA and ρNPB) did not significantly differ in mussels from the two sites, naphthyl-derived (αNA and αNB) associated CE-activities were impacted in those chronically exposed to PAHs ($p<0.05$) and were highly correlated between them ($r = 0.702$; $p<0.05$; $n=16$). Since the different substrates are likely to inform on specific CE isoforms (Wheelock et al., 2008), the particular responses of naphthyl-substrates suggests that they would be more adequate for PAH monitoring. To the best of our knowledge, this is the first time to relate PAH exposure to CE-dependent activities although in mammals CE responses are modulated by multiple xenobiotic receptors including AhR which is particular for PAHs (Zhang et al., 2012). However, it cannot be ignored that other chemicals present (e.g metals) could be responsible for CE inhibition using these two particular substrates). Metals are regarded as B-esterase inhibitors in aquatic species, mostly from *in vitro* and fish studies (Frasco et al., 2005; Vieira et al., 2009; Oliva et al., 2012) although under field conditions results are not so conclusive. Co-occurrence of higher loads of metals and petrogenic PAHs has been described in other harbours of the Iberian Peninsula (Perez-Fernandez et al., 2019).

Correlations between enzyme activities in mussels chronically exposed to PAHs

Not only there were differences in terms of total enzymatic activity in mussels collected at the two sites (Table 2), but correlations between biomarkers also differed in PAH-free and polluted sites considering a larger number of individuals from the polluted site (n=22) since size did not affect these enzymatic activities and recent data from our group for the Ebre Delta site (Table 3). Former studies with mussels collected

255 in the PAH-free Ebre Delta waters (with Σ13 PAHs levels < 10 ng/g w.w.) revealed a
256 good correlation between biomarkers including CE-related measures using several
257 substrates (Dallares et al., 2018). Wild mussels collected from the chronically PAH-
258 polluted waters of the Barcelona harbour were also used to assess associations between
259 the same biomarker activities. The lack of agreement of these formerly observed
260 correlations (Dallares et al., 2018) with present results in chronically polluted mussels
261 could supports the particular modulation by the chemicals present in the harbour waters
262 to the different CE-isoforms. Among the antioxidant defences, CAT, GR and GSTs
263 were positively correlated among each other (r=0.428-0.556) but GPX was negatively
264 correlated with GR (r=-0.931) and GSTs (r=-0.470). To the best of our knowledge we
265 are only aware of one study relating *in vivo* PAH exposure in fish (through water
266 accommodated fraction-WAF) and CEs (using ρNPB as substrate) as well as further
267 exposure *ex vivo* in fish liver slices (de Anna et al., 2018). In this former study with
268 rainbow trout, CE activity was inhibited by 42% in fish exposed in vivo to WAF for 48h
269 and the inhibitory action was further confirmed in *ex vivo* exposures. In another study
270 with Atlantic killifish, inhabiting sites chronically polluted sites by PAHs, a low
271 CYP1A activity expressed in this fish was associated to resistance to organophosphorus
272 (OP) pesticides since the oxon metabolites were not formed (Clack and Di Giulio 2012),
273 but no reference to CE activity was made. To the best of our knowledge, CE regulation
274 by specific nuclear receptors has been studied only in rodents, with the involvement of
275 the AhR in modulating certain CE isoforms (Zhang et al., 2012). Since mussels express
276 low to undetectable CYP1A-related EROD activities but express other nuclear receptors
277 involved in xenobiotic metabolism (Raingeard et al., 2013), the chronic action of PAHs,
278 and other chemicals of environmental concern on CEs activity and receptors modulation
279 in bivalves deserves investigation. Mussel CE inhibition (using naphthyl substrates) in

those collected in the harbour cannot be ascribed to PAHs or metal exposures (or both) and further independent exposures are needed to explore the mechanistic action of these environmental chemicals.

S9 determinations and CEs as alternative to CYPs components and activities

Antioxidant enzymes are mostly cytosolic but can also be measured in the post mitochondrial S9 fraction while the different CEs are either soluble or membrane bound so they can be analysed in the three fractions (cytosol, S9 and microsomes). In all cases, these parameters showed good reaction rates and could be confidentially be measured. However, lower standard deviation and coefficient of variation on the S9 measures makes this easily obtained fraction more adequate (Table 4). Unspecific Cyt P450-reductases and CYP-related catalytic measures could only be reliably measured the microsomal fraction as they are tightly associated to endoplasmic reticulum membranes. Thus, given that we aim to simplify monitoring protocols (by using, for example, a single subcellular fraction), these measurements were excluded from site comparisons. We could, however, observe that the activities (in nmol/min/mg prot) in mussels collected from the Ebre Delta followed the order NADPH cit c red. <NADH cit c red. <NADH ferricyanide reductase as it was seen in other studies using invertebrates, including mussels (Sole and Livingstone, 2005). CYP-related activities were only attempted in the microsomal fraction where these membrane-bound proteins are located. Eight fluorometric substrates, among those commonly used for measuring CYP-related activities in fish, were tested. No CYP-related activity was detected when using ER, BzRs, PR, MR and CEC as substrates (see materials and methods section). Measurable fluorescence readings were only obtained when using DBF and BFC as substrates,

304 although these measures were much lower than in fish (Sole et al., 2012). Using
305 ketoconazole, a broad CYP inhibitor, at the concentrations of 0.1, 1 and 10 μM, BFC- or
306 DBF-related activities were not affected (data not shown). This contrasts with previous
307 results in fish, where a ketoconazole concentration of 10 μM causes a reduction in
308 activity when using as substrates BFC (up to 70%) or DBF (68%) in fish (Koenig et al.,
309 2013). Some studies report CYP related activities in bivalves using DBF (Aguirre-
310 Martinez et al., 2015; Almeida et al., 2015; Pereira et al., 2012). However, to our
311 knowledge, none of these bivalve studies confirmed the CYP nature of the measures by
312 using stablished CYP inhibitors or discuss the fact that maybe other enzymes could be
313 responsible for metabolites formation. Using the CYP-substrates traditionally used in
314 fish, our observations support the lack of comparable CYP catalytic activities in *M.*
315 *galloprovincialis*, despite the existence and purification of a CYP protein and its
316 immunodetection in the marine mussel *M. edulis* (Porte et al., 1995; Shaw et al., 2004)
317 or CYP-related genes being identified in *Mytilus edulis* (Zanette et al., 2013).

318 Because of all the above (i.e. CYPs requiring working with a unique subcellular
319 microsomal fraction and the uncertainty of results being attributable to CYPs activities
320 in bivalves), we suggest that biomarkers of pollution (antioxidant defences) can be
321 confidentially measured in S9 and include CE determinations in this same fraction in
322 future monitoring programs using bivalves. This would have the advantage of using one
323 single cellular fraction (S9), where all the biomarkers could be determined. CEs would
324 be a good alternative to reductases since they are also general metabolic markers, as
325 suggested by Satoh and Hosokawa (1998), that respond to many xenobiotics and seen in
326 studies using the fish *Solea senegalensis* exposed to chemicals of environmental
327 concern (Sole et al., 2014).

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Validation of biomarker assays using commercial proteins

The use of commercial purified proteins showed good data linearity for most of the selected biomarkers measures, following the particularity of the corresponding protocols and respecting the protein ranges shown in Table 1. Since in biomarker studies it is often difficult to compare results with other works due to the use of different methodologies, nature of the extraction buffers, centrifugation steps and activities expression, the use of protein standards is highly advisable for quality assurance and comparison of results. Even within a same research group and using the same methodology this procedure is recommended in order to validate the quality and reproducibility of the multi-well measures in large scale comparative studies. This is a well-established practice in other fields such as chemistry, and the inclusion of purified protein in biomarkers studies is, to our criteria, a good methodological practice.

Conclusions.

CYP-related measurements in mussels, using the most common fish fluorometric substrates, are questionable. The present results suggest other phase I related parameters, such as CE activities, as alternatives. This is supported by the fact that: i) they can be measured in S9 fractions (so they could be carried out along with the other biomarkers commonly considered in pollution monitoring) and that ii) CE hydrolysis rates using any of the four proposed substrates (ρNPA, ρNPB, αNA and αNB) are high in mussel digestive glands and they are responsive to PAHs exposure (as seen in the present study) as well to pesticides and other xenobiotics. In the light of these evidences, we propose to include CE measurements as biomarkers, as well as the

352 traditional antioxidant and biotransformation responses when using mussels as sentinels
353 and the inclusion of purified proteins for quality assurance.

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568

Table 1. Purified protein standards from Sigma-Aldrich. Abbreviation of enzyme activities and substrates as in the Material and Methods section. CE1 Human carboxylesterase 1, CE2 Human carboxylesterase 2.

Enzyme Assay	Sigma-Aldrich code	substrate	Recommended enzyme dilution in assay	Protein range mg/ml	slope	R^2
CAT	SRE0041	H_2O_2	1/20	0.1-0.5	0.010	0.780
GR	G3664	GSSG	1/400	0.02-0.08	0.006	0.987
GPX	G6137	H_2O_2	1/20	0.03-0.18	0.007	0.995
GST	G6511	CDNB	1/80	0.03-0.31	0.006	0.996
CE1	E0162	ρNPA	1/100	0.25-2.50	0.079	0.999
		ρNPB	1/300	0.03-0.25	0.225	0.998
		αNA	1/100	0.13-1.25	0.013	0.861
		αNB	1/200	0.06-0.62	0.216	0.928
CE2	E0412	ρNPA	1/20	0.63-6.25	0.018	0.996
		ρNPB	1/80	0.16-1.56	0.067	0.999
		αNA	1/20	0.63-6.25	0.017	0.991
		αNB	1/40	0.13-3.13	0.041	0.995

Table 2. Mussel *Mytilus galloprovincialis,* from two locations from the North-western Mediterranean: aquaculture region in the Ebre Delta (pristine site) and Barcelona harbour (PAH-polluted site). Enzymatic activities (assessed in S9 fractions) are expressed in nmol/min/mg prot except CAT in μmol/min/mg prot (n=8). Σ PAHs in ng/g w.w. Contrasts by Student's *t*-test, significant p value in black ($p<0.05$). All abbreviations are used as defined in the Materials and Methods section.

		Ebre Delta mussels	Harbour mussels	
	Size (cm)	4.6 ± 0.21	4.7 ± 3.0	
	Σ PAHs	9.0	565.0	
	Biomarkers			p value
Antioxidants	CAT	34.4 ± 2.7	49.8 ± 7.7	0.08
	GR	9.0 ± 0.8	12.2 ± 1.1	**0.03**
	t-GPX	22.3 ± 1.7	15.8 ± 1.3	**0.01**
	GSTs	40.1 ± 3.3	55.8 ± 5.2	**0.02**
Carboxylesterases	ρNPA-CE	40.7 ± 1.9	38.3 ± 2.3	0.44
	ρNPB-CE	41.1 ± 1.9	45.2 ± 5.0	0.57
	αNA-CE	68.4 ± 2.1	54.6± 2.9	**<0.01**
	αNB-CE	54.4 ± 2.6	38.9 ± 3.5	**<0.01**

Table 3. Pearson correlation coefficient between biomarker activities measured in the S9 fraction of the liver in mussels from the Ebre Delta[1] (n=40) and Barcelona harbour (n=22). Only significant correlations are indicated. $p<0.05$. n.a. not available and n.s not significant. Abbreviation of enzyme activities and substrates as in the Material and Methods section.

Ebre Delta	Antioxidants				Carboxylesterases			
N=40	CAT	GR	GPX	GST	ρNPA	ρNPB	αNA	αNB
CAT		n.a	n.a	n.a	n.a	n.a	n.a	n.a
GR			0.595	0.484	0.740	0.731	0.688	0.676
GPX				0.469	0.545	0.581	0.502	0.574
GST					0.425	0.493	0.392	0.466
ρNPA						0.837	0.939	0.813
ρNPB							0.732	0.820
αNA								0.819
αNB								

[1] Calculated from data from Dallarés et al., 2018.

Harbour	Antioxidants				Carboxylesterases			
N=22	CAT	GR	GPX	GST	ρNPA	ρNPB	αNA	αNB
CAT		n.s	n.s	0.428	0.499	n.s	0.549	n.s
GR			-0.931	0.556	0.610	0.451	0.561	0.599
GPX				-0.470	-0.573	n.s	0.475	-0.531
GST					0.713	0.451	0.600	0.569
ρNPA						n.s	0.913	0.608
ρNPB							n.s	0.718
αNA								0.458
αNB								

Table 4

Table 4. Enzyme activities in the different subcellular fractions (S9, cytosol and microsomes) of mussel's digestive gland. Activity in nmol/min/mg prot except for CAT which was in μmol/min/mg prot. Data expressed as mean ± sem (n=6). The coefficient of variation of the measures indicated in brackets. Abbreviations as in the Material and Methods section. n.a = not analysed. n.m= not measurable.

Activities	S9	cytosol	microsomes
Antioxidant			
CAT	75.15 ± 2.18 (7.1)	82.64 ± 5.72 (16.9)	n.a
GR	16.65 ± 0.66 (9.7)	19.76 ± 1.51 (18.7)	n.a
t-GPX	12.51 ± 0.14 (2.8)	7.11 ± 0.52 (17.9)	n.a
GST	68.68 ± 1.55 (5.5)	78.90 ± 6.35 (19.7)	n.a
Carboxylesterases			
ρNPA-CE	53.50 ± 2.40 (11.0)	69.35 ± 8.10 (28.6)	31.37 ± 2.83 (22.1)
ρNPB-CE	117.9 ± 3.39 (7.0)	123.7 ± 13.9 (27.6)	84.60 ± 8.43 (24.4)
αNA-CE	105.8 ± 5.64 (13.0)	121.5 ± 14.1 (28.4)	62.09 ± 5.79 (22.8)
αNB-CE	121.9 ± 4.81 (9.7)	140.7 ± 14.4 (25.1)	85.4 ± 10.2 (29.3)
Reductases			
NADPH-cyt c.	n.m	n.a	9.12 ± 1.05
NADH-cyt c.	n.m	n.a	17.96 ± 2.43
NADH-ferryc.	n.m	n.a	272.3 ± 27.6